ligation, suggesting that a mismatch at the 3' ligation junction imposes substantial constraints on the ability of *Thermus* ligases to close the nick, thereby limiting the turnover of DNA-adenylate intermediate into ligated product and free AMP (the third step of ligation cycle). The effects of moving the T/G mismatch one base-pair away from the ligation junction was further examined. The rates of ligation with a T/G mismatch at the penultimate 3' end in general improved several-fold as compared with the T/G mismatch at the 3' end of the ligation junction. However, the ligation rates were still much slower than those of match ligation, emphasizing the importance of nucleotide complementarity near the ligation junction as well as the ultimate critical role of the perfect base-pair at the 3' end in controlling ligation reaction. Consequently, the ligation fidelity when the mismatch was at the second position from the 3' side (ligation fidelity 2) was lower than that when the mismatch was located immediately at the ligation junction. It is noteworthy that the *Tsp.* AK16D enzyme maintains extremely high fidelity (1.1×10^3) even when the mismatch is at the penultimate position, further underscoring the discriminative power of this new *Thermus* ligase.

Example 14 - Thermostable DNA Ligase Fidelity In The Presence Of Mn^{2+}

Many enzymes such as DNA polymerase and restriction endonucleases demonstrate relaxed specificity when Mn^{2+} is used as the metal cofactor. The influence of metal ion substitution on ligase fidelity has not been fully investigated although it is known that Mn^{2+} can be used as an alternative metal cofactor for a ligation reaction ((Ho, et al., J Virol, 71(3):1931-1937 (1997) and Cheng, et al., Nucleic Acids Res, 25(7):1369-1374 (1997), which are hereby incorporated by reference). The reaction rates of the match and mismatch ligation for *Tsp.* AK16D ligase and *Tth* ligase were determined. As shown in Table 3, the match ligation rates were higher with Mg^{2+} than with Mn^{2+} (Table 3), in agreement with the consistent high ligation rate under various Mg^{2+} conditions (Figure 4-6).

Table 3. DNA ligase fidelity with Mn^{2+} [a]

Ligase	Concentration (nM)	Initial rate of C-G match (fmol/min)	Initial rate of T-G mismatch (fmol/min)	Ligation fidelity
Tth-wt	1.25	2.6×10^{1}	3.7×10^{-1}	7.0×10^{1}
Tsp. AK16D	12.5	9.5×10^{1}	1.1×10^{-1}	8.6×10^{2}

[a] Reaction conditions were identical to those in Table 2, except that 10 mM Mn^{2+} was used in place of Mg^{2+}. Ligation fidelity was defined as the ratio of Initial Rate of C-G match divided by Initial Rate of T-G mismatch at 3'-end. Results were calculated as the average of at least two experiments.

The mismatch ligation rate of *Tth* ligase was about six-fold higher with Mn^{2+} than with Mg^{2+} while that of *Tsp.* AK16D ligase was about 4-fold higher. Thus, as with other previously studied DNA enzymes, DNA ligases also demonstrate relaxed specificity when Mg^{2+} is substituted with Mn^{2+}. As a result, the fidelity factors of *Tth* ligase and *Tsp.* AK16D ligase were reduced 12- and 6-fold, respectively (Tables 2-3). Remarkably, the *Tsp.* AK16D enzyme retains 12-fold higher fidelity against mismatch ligation than the *Tth* enzyme. In contrast to using Mg^{2+} as the metal cofactor, *Tth* ligase did not generate DNA-adenylate intermediate during 3'T/G mismatch ligation with Mn^{2+}. This observation suggests that the nick closure of a 3'T/G mismatch by the *Tth* enzyme is accelerated with Mn^{2+}. On the other hand, the *Tsp.* AK16D enzyme accumulated DNA-adenylate intermediate during 3'T/G mismatch ligation with either Mg^{2+} or Mn^{2+}. These results indicate that the nick closure of a 3'T/G mismatch with Mn^{2+} by *Tsp.* AK16D DNA ligase remains as the rate-limiting step, which accounts for the higher fidelity of this enzyme.

Studies on *Tth* DNA ligase has deepened understanding of thermostable ligases and has reaffirmed the common theme of ligation – adenylation of ligase at the KXDG motif (Luo, et al., Nucleic Acids Res, 24(15):3079-3085 (1996), which is hereby incorporated by reference). This study reveals that *Thermus* ligases may differ from each other as to substrate specificity despite their highly identical primary protein sequences. A highly homologous structure can be anticipated from various *Thermus* ligases, but subtle local environments may dictate the probability of accepting a particular mismatch as the substrate. The fidelity of the

Thermus ligases may be determined by multiple domains, multiple motifs and/or multiple sequence elements. In comparison of *Tth* and *Tsp.* AK16D ligases, one can find that although K294R (in an identical local environment, see Figure 1B) enhances the fidelity of *Tth* ligase (Luo, et al., Nucleic Acids Res, 24(15):3071-3078 (1996), which is hereby incorporated by reference), *Tsp.* AK16D ligase with a K in this position can still demonstrate superior mismatch discrimination. Additional sequence elements remain to be uncovered. The R substitution at the adjacent position to the KXDG motif may have an effect on the *Tsp.* AK16D ligase's specificity, because studies on *Chlorella* ligase has emphasized the importance of occupying AMP binding pocket for nick recognition (Sriskanda, et al., Nucleic Acids Res, 26(2):525-531 (1998)). The accumulation of DNA-adenylate intermediate with some divalent metal ions by *Tsp.* AK16D ligase asserts that the nick closure step of a ligation reaction can be sensitive to the selection of metal ions, gapped substrates and mismatch substrates. More structural and functional studies on *Tsp.* AK16D ligase could reveal how this enzyme achieves high fidelity with different substrates and different metal ions.

Although the invention has been described in detail for the purpose of illustration, it is understood that such details are solely for that purpose and that variations can be made therein by those skilled in the art without departing from the spirit of the scope of the invention which is defined by the following claims.